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Differential Detection of Soluble Enzymes Specific to a Rodent Malaria Parasite, *Plasmodium berghei*, by Electrophoresis on Polyacrylamide Gels

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ABSTRACT: In plasma samples of mice infected with a rodent malaria parasite, NK 65 strain of *Plasmodium berghei*, decreases in total proteins and albumin were noticed whereas increases in enzyme activities were significant in LDH, GOT, and GPT. By means of horizontal electrophoresis on 5 % polyacrylamide gel plates, plasma, hemolysates of normal or malaria-infected mice, and cell-free extracts of the parasite were compared for various enzyme activity bands. Of several enzymes examined, the parasite extracts contain the following enzymes of which electrophoretic mobilities differ from those of the host blood system: LDH, MDH, GDH, catalase, GOT, and esterases. With the exception of the esterases, which migrate rather rapidly towards the anode, all the parasite specific enzyme bands have relatively slow mobilities around an area between the start line and plasma ChE position. No detectable band of parasite-specific G6PDH has been found except for the RBC-specific bands, suggesting that initiation of the pentose phosphate cycle in the parasite substantially depends upon the G6PDH of the host RBC.

To support their rapid growth, nuclear division and multiplication, energy generation of erythrocytic forms of malaria parasites must largely depend on metabolism of the host blood system. Investigations on qualitative or quantitative differences in enzymatic features between the host and the parasite are worth-while to infer the parasite-host

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Abbreviations used are: LDH, lactate dehydrogenase; MDH, malate dehydrogenase; GDH, glutamate dehydrogenase; SDH, succinate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; GOT, aspartate aminotransferase; GPT, alanine aminotransferase; LAP, leucine aminopeptidase; ChE, cholinesterase; RBC, erythrocyte(s); WBC, leukocyte(s); Hb, hemoglobin; Tris, tris(hydroxymethyl) aminomethane; BIS, N, N′-methylenebisacrylamide; TEMED, N, N, N′, N′-tetramethylethylenediamine; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; PMS, phenazine methosulfate; NBT, Nitro blue tetrazorium; EDTA, ethylenediamine tetraacetic acid (2Na salt); BPB, bromophenol blue; G-6-P, glucose-6-phosphate; TCA, tricarboxylic acid; PBS, phosphate-buffered physiological saline; M.W., molecular weight

relationship from a biochemical viewpoint. In relation to malarial infection, numbers of enzymes in blood system have been investigated enzymologically, radioisotopically, cytochemically or electrophoretically by various workers (Peters, 1969; 1970). Available informations on the biochemistry of malaria, however, are still fragmentary, inconclusive or incomplete to draw a definite conclusion, and sometimes even being contradictory each other. The reasons for such a confusion are probably due to differences in materials used and/or methods employed by different investigators. Contamination of the host blood cells or their fragments, such as platelets, WBC and RBC cytoplasm (as food vacuoles within the parasite cytoplasm), into so-called "malaria parasite fraction" might be one of the major suspicious factors, too. In some cases, therefore, it is rather difficult to define whether or not reported enzyme activities in malaria parasites have been due to their own enzymes.

The recent development of polyacrylamide gel electrophoresis is of great advantage for distinguishing an enzyme activity band from other isoenzymes by differences in molecular sizes and ionic charges of proteins. Disc electrophoresis has great resolving potentiality for the individual sample but comparisons between separate gels are not convenient. Thus, in the present work, attempts have been made to compare electrophoretic mobility of several soluble enzymes of the host blood system together with those of malaria parasites on a single polyacrylamide gel plate in the same run so that an enzyme band of parasitic origin can be differentiated directly from that of host origin by their migration behaviors under an electric field. At least 6 enzymes of parasitic origin have successfully been distinguished from those of host origin.

MATERIALS AND METHODS

Malarial Strain: The malaria parasite used is NK 65 strain of *Plasmodium berghei* VINCKE and LIPS obtained from Dr. M. Suzuki, Institute of Medical Science, University of Tokyo. In earlier stages of the present work, a strain (the name was not available) obtained from Dr. Q. M. Geiman, Stanford University School of Medicine, was used. The parasites were maintained by serial blood passage in laboratory albino mice.

Sample Preparation: The mice, usually males weighing 18–23 g, were inoculated intraperitoneally with malaria-infected mouse blood, and when parasitemia had reached 40–50% (usually 5 days later) blood was collected from ether-anesthetized mice by cardiac puncture into an EDTA-treated test tube. In some cases where enzymes to be tested were sensitive to the metal chelating agent or only small amounts of fresh samples were required, tail blood was collected into a heparinized micro-hematocrit capillary tube. Plasma samples were separated by centrifugation at 2,000 × g for 10 min in a cold room or at 10,000 × g for 5 min by a hematocrit centrifugator at room temperature. The packed blood cells were suspended in about 10 volumes of an ice-cold 0.85% NaCl solution containing 4% dextran (M. W. 200,000–230,000) then slowly centrifuged at 10 × g for

10 min or the tubes were just stood in an ice bath for 1 hour. The supernatant, mainly consisted of platelets and WBC, was removed by a pipet, and the precipitate was resuspended into about 10 volumes of an ice-cold 0.85% NaCl solution buffered with 0.02M sodium phosphate, pH 7.4 (PBS). The RBC suspension was then passed through a column packed with dry cellulose powder (Whatman Column Chromedia CF 12) in order to further remove any contaminated WBC from the sample (Fulton and Grant, 1956; Richard and Williams, 1973). The resultant eluate contained practically no WBC whereas removal of lymphocytes was still incomplete. The RBC fraction was sedimented at 1,500 × g for 10 min, and the packed RBC fraction was lysed by suspending into 10 volumes of saline containing 0.5% saponin at 30°C then followed by centrifugation at 1,500 × g for 10 min at room temperature. Subsequently, the parasites freed from the host RBC were washed 4 times with the ice-cold PBS by centrifugations at 1,500 × g for each 10 min. At this stage of the procedures the parasite preparation still possessed the infectious ability. The final packed parasite fraction was then stored in a deep freezer at −70°C until used. Cell-free extracts of the parasites were obtained by at least 2 cycles of freezing-and-thawing followed by homogenization with a Teflon-glass homogenizer in an ice bath and centrifuged at 20,000 × g for 30 min at 0°C. Finally the extracts were concentrated to an original volume of packed parasites by a disposable membrane filter kit, Minicon (Amicon Corp.). Hemolysates from normal or infected RBC were obtained by suspending the packed RBC into 5−10 volumes of glass distilled water followed by centrifugation.

Quantitation of Plasma Proteins and Enzymes: Plasma samples freshly prepared from normal or malaria-infected mice (5−6 days after inoculation) were compared for amounts of proteins and activities of some enzymes by a commercial clinical examination kit, UNIKIT Rapid Blood Analyzer System (Chugai Pharmaceutical Co., Ltd.). The items tested were as follows: total protein, albumin, cholesterol, LDH, GOT, GPT, and LAP.

Electrophoresis: 5% polyacrylamide gel plates, 0.9 mm thickness, were prepared by mixing the following stock solutions in 2 : 1 : 1 volume ratio in this order (modification of the method of Ogita, 1965):

A.	Acrylamide, monomer	19.0	g
	BIS	1.0	g
	Distilled water	to 200	ml
B.	Tris	9.12	g
	1N HCl	12.0	ml
	TEMED	1.0	ml
	Distilled water	to 100	ml
C.	Ammonium persulfate	120	mg
	Distilled water	100	ml

The stock solutions A and B can be stored without the loss of polymerizing capability in dark bottles in a refrigerator for at least one month. For the solution C, however, it is desirable to prepare fresh every week. To detect catalase-peroxidase bands, measured

amounts of starch were added into the gel mixture before polymerization. An electrode buffer used for either cathode or anode was a 0.3M boric acid−0.05N NaOH solution, pH 8.3. Horizontal electrophoresis was carried out in a refrigerator at a constant gradient of voltage: 10 V/cm for oxidoreductases and 15 V/cm for proteins or other enzymes. A distance between filter-paper wicks at both electrodes was routinely 7.0 cm, and a distance between sample ditches and an edge of the cathodal wick was 1.0 cm. A diagram of the set up for the gel plate electrophoresis is given in Figure 1. A small droplet of a 0.05% BPB solution was utilized as a visible marker for the discontinuous buffer front. When the front marker had moved a 5 cm distance from the original start line towards the anode, the electrophoretic run was terminated. Disc electrophoresis described by Davis (1964) and modified by Dietz *et al.* (1970) for separating LDH isoenzymes were also performed.

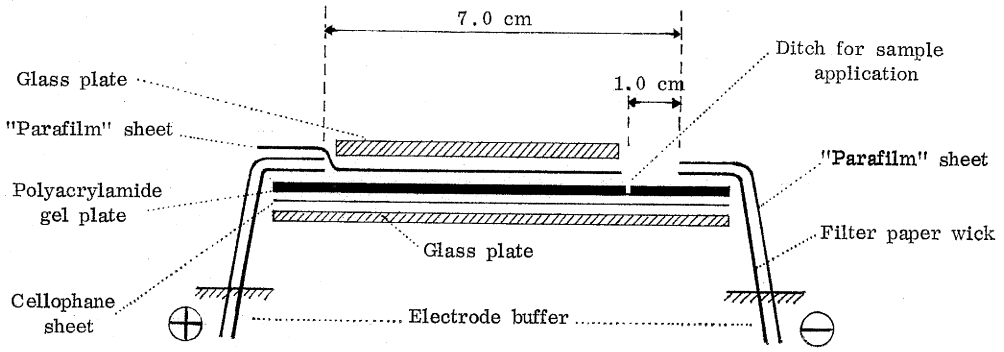

Fig. 1. Diagram of set up used for horizontal electrophoresis.

Detection of Proteins or Enzyme Activity Bands: Protein bands were stained by 0.5% Amido Black 10 B in a mixture of methanol−water−acetic acid (5 : 4 : 1), and destained with 7% acetic acid at 50−60°C. Incubating mixture solutions for staining enzyme activity bands were prepared in dark just before the termination of the run, and unless otherwise stated gels were incubated at 37°C. The darkness during the preparation of staining solutions and the incubation period is quite important especially for the detection of dehydrogenases and esterases. Freshly prepared incubation mixtures should be pre-warmed just prior to the incubation in a water bath at 37°C. This procedure is quite effective especially for dehydrogenases to gain more intensified activity bands because usually the buffer stock solutions are stored in a refrigerator. In the case of esterases or catalase-peroxidase, a cold buffer solution is rather preferable to use as an incubation medium without warming. After detecting the activity bands or protein bands, the gel was sandwiched between two cellophane sheets which were immersed in advance in 0.5−1% glycerol, attached to a glass plate, then allowed to form a dried film in air. Care must be taken to avoid leaving any air bubbles between the sheets.

Recipes used for visualizing enzyme activity bands are listed below:

Lactate dehydrogenase (LDH, EC 1.1.1.27)	0.1M Phosphate buffer, pH 7.4	50 ml
	60% Sodium DL-lactate	3 ml
	NAD	20 mg
	NBT	15 mg
	PMS	1 mg
	KCN	15 mg
Malate dehydrogenase (MDH, EC 1.1.1.37)	Same as LDH, but substitute 300 mg of sodium DL-malate for lactate.	
Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49)	0.1M Tris-HCl buffer, pH 8.5	50 ml
	Sodium G-6-P • $3H_2O$	50 mg
	NADP	20 mg
	NBT	15 mg
	PMS	1 mg
	EDTA	20 mg
	$MgCl_2 \cdot 6H_2O$	50 mg

Fresh blood samples fortified with NADP and 2-mercaptoethanol (0.1 mg and 1 μl per ml, respectively) were used.

Glutamate dehydrogenase (GDH, EC 1.4.1.4)	0.1M Tris-citrate buffer, pH 8,5	50 ml
	Sodium L-glutamate	100 mg
	NADP	30 mg
	NBT	15 mg
	PMS	1 mg
Catalase (EC 1.11.1.6) & Peroxidase (EC 1.11.1.7)	A : 2% KI in 2% acetic acid	
	B : 0.03% H_2O_2	

Weigh an appropriate amount of soluble starch (final concentration, 0.5%); dissolve into small amounts of water in a beaker; heat it shortly until the solution becomes transparent and the volume of water is reduced as small as negligible; then prepare a gel mixture in this beaker as usual. Immediately following electrophoresis, immerse gels into an ice-cold solution of the 0.3M borate buffer, pH 6.5, for 40 min. Then, keep gels in the solution A for 1 min; wash with cold water 3 times; and immerse gels into the solution B until white (catalase) and dark blue (peroxidase) bands appear against a blue-violet background. Since the color intensity changes rapidly, photo pictures for record should be taken as soon as possible when the color intensity has reached an optimum.

Aspartate aminotransferase (GOT, EC 2.6.1.1)	A :	0.1M phosphate buffer, pH 7.4	50 ml
		L-Aspartate	200 mg
		2-Oxoglutarate	20 mg
		Pyridoxal phosphate	20 mg
	B :	0.1M Phosphate buffer, pH 7.4	10 ml
		Fast blue B (or Fast violet B)	50 mg

Incubate gels in the mixture A at 37°C for 10 min, then add the solution B and further incubate for 30−60 min at 37°C in dark. When dark blue or violet bands appear, wash the gel with water several times to remove excess brownish background stain.

Esterases (EC 3.1.1.−)

A :	α-Naphthyl acetate	50 mg
	Acetone	3 ml
B :	0.1M Phosphate buffer, pH 6.8	40 ml
C :	0.1M Phosphate buffer, pH 6.8	20 ml
	Fast blue B (or Fast violet B)	30 mg

Place the substrate (A) in an acetone-proof reaction vessel in dark and dissolve it with acetone followed by the cold phosphate buffer (B); immerse a gel plate immediately into this emulsion at room temperature; 2−3 min later, add the solution C and keep dark. Occasional gentle aggitations of the container were effective to prevent the adhesion of the gel onto the bottom of the container. Usually within 5−10 min, dark brown-violet bands appear at room temperature. When the color intensity has reached the most desirable one, still continue the incubation for 3−5 min more; then wash the gel rapidly with water 2−3 times and fix it in 7% acetic acid. Some excess dark background may be reduced during the fixation. Exposure to light should be minimized during all the procedures, otherwise it may cause the darker background.

RESULTS

Plasma Protein and Enzyme Levels

Results of quantitative analysis on plasma proteins and some enzyme activities are summarized in Table 1. Except for measurement of LDH levels, undiluted plasma samples were used. It is of interest to note that the plasma LDH levels even in normal mice are extremely higher than a normal range of LDH levels in human plasma. Thus in cases of mouse plasma LDH, plasma samples were diluted 5- or 10-fold with the PBS. Decreases in total protein and albumin levels in malaria-infected mouse plasma (parasitemia at 7th day, 60−75%) were observed in accordance with results reported by previous investigators (Sadun *et al.*, 1965, 1966; Tella and Maegraith, 1965; Sizaret *et al.*, 1971), although the former was not significant at the 5% level. On the other hand, statistically highly signifficant increases in enzyme activities of LDH, GOT and GPT were shown in plasma of malaria-infected mice.

Electrophoretic Patterns

In earlier stages of this investigation, disc electrophoresis was mainly employed and resulted in resolution of sharp protein bands within each gel. However, in later

Table 1. Quantitative comparison of plasma biochemical values between normal and malaria-infected mice

Item	Mice	No. of test	Mean and 95% fiducial limits	Unit
Total protein	Normal	18	5.21 ± 0.33	g/100 ml
	Infected	16	4.73 ± 0.27	〃
Albumin	Normal	18	3.31 ± 0.13	〃
	Infected	16	2.97 ± 0.13*	〃
Cholesterol	Normal	11	100.4 ± 12.3	mg/100 ml
	Infected	12	108.7 ± 15.3	〃
LDH	Normal	21	2935 ± 282	Wroblewski
	Infected	16	13050 ± 1624**	〃
GOT	Normal	14	93.1 ± 10.4	Karmen
	Infected	14	257.4 ± 35.0**	〃
GPT	Normal	15	38.2 ± 7.1	〃
	Infected	15	120.5 ± 37.2**	〃
LAP	Normal	12	103.8 ± 13.4	Goldberg-Ruenburg
	Infected	13	127.2 ± 13.8	

*) Significantly different from the normal control at the 5% level.
**) Significantly different from the normal control at the 1% level.

horizontal electrophoresis on gel plates was mostly carried out to compare exact mobilities of similar bands to each other. Electrophoretic patterns of proteins in the host and parasite samples on 5% polyacrylamide gel plates are schematically illustrated together with those of enzyme activity bands in Figure 2, where position of the Hb is shown as a reference protein so that relative mobility of each band can be easily compared.

Proteins

Mouse plasma : Separation of plasma proteins by disc electrophoresis was effective and at least 16 bands were detected. Whereas there were considerable individual variations in protein patterns (since no inbred mouse line was used), no consistent qualitative difference was observed between normal and malaria-infected mice. Resolution of plasma proteins by horizontal electrophoresis on gel plate was less sensitive but still 10−12 bands could be recognized. All the mouse plasma proteins were not immunologically identified, but on the basis of analogy to human plasma proteins, several characteristic proteins in the mouse plasma could be recognized to be prealbumin, albumin, Hb, transferrin, alpha-2 macroglobulin, and so on.

P. berghei extracts : By disc electrophoresis, at least 11 protein bands were detected in accordance with results reported by several workers (Chavin, 1966; Corradetti *et al.*, 1971; Desowitz *et al.*, 1966; Spira and Zuckerman, 1966). Horizontal electrophoresis also resulted in the separation of 8−9 bands. The major one migrated with the buffer front, and other minor bands were distributed from the post-albumin area to the original line. Since no immunological test has been performed, it is very difficult to

reveal whether other minor bands found in the parasite extract fraction are realy parasitic origin or just due to host RBC components.

LDH

Normal mouse plasma contains all the known 5 isoenzymes of LDH, among which LDH-5 is the major one. Extracts of malaria parasites possessed an LDH band distinct from the host LDH isoenzymes, *i. e.*, the parasite LDH band had a slower electrophoretic mobility than any known LDH bands of the host including that of spermatozoa (Allen, 1961; Goldberg, 1965). The mobility of LDH band found in mouse RBC is similar to but slightly slower than that of the plasma LDH-5. In addition to usual LDH bands, plasma samples obtained from heavily infected mice often contained the parasite LDH and RBC-LDH which appeared to be as if an intense tailing of the plasma LDH-5 band. The significant increase of LDH activity in plasma of malaria-infected mice is accountable for the release of the enzyme into blood stream both from the infected host RBC and from the parasites. The parasite extracts also often possessed not only the parasite-specific LDH but also the host LDH-5 band even after thorough washing of the parasites freed from the host cells. Thus this may not be a technical contaminant of the host RBC component but may be released from "food vacuoles" within the parasite cytoplasm. Mobility of the malaria parasite-specific LDH band corresponded to an area around the plasma ChE band and to one of the protein bands detected in the parasite extracts. Disc electrophoresis of a mixture of host and parasite samples by the modified technique of Dietz *et al.* (1970) thus effectively demonstrated all the 6 separated LDH bands in a single gel. Phisphumvidhi and Langer (1969), using NYU-2 strain of *P. berghei*, reported the presence of a distinct LDH band in the parasite extracts. The illustrated curve for LDH of the mouse RBC by a scanning densitometer, however, is completely different from results of the present work.

MDH

In normal mouse plasma, usually only one major MDH band, which migrated faster than the transferrin band but slower than the Hb, and a minor band near the buffer front could be detected. Hemolysates of the host RBC showed one major band of which mobility corresponded to the transferrin. In malaria-infected mouse plasma, the major MDH band shifted to the transferrin area. Under the routine alkaline buffer condition employed, the parasite extracts possessed an additional major MDH band of which mobility was corresponding to that of the malarial LDH band. By using acidic buffer conditions, however, the parasite LDH and MDH bands could successfully be separated each other: for example , 0.03M Tris-maleate gel buffer, pH 5.4, or 0.05M Na_2HPO_4-citrate gel buffer, pH 3.8, and 0.1M Tris-maleate electrode buffer, pH 6.5; where the parasite LDH migrated towards the anodal side and the MDH bands towards the cathodal side. In the latter cases, the major MDH band separated into at least 2 distinct bands. More extensive studies should be performed on the malarial MDH in future. Under the

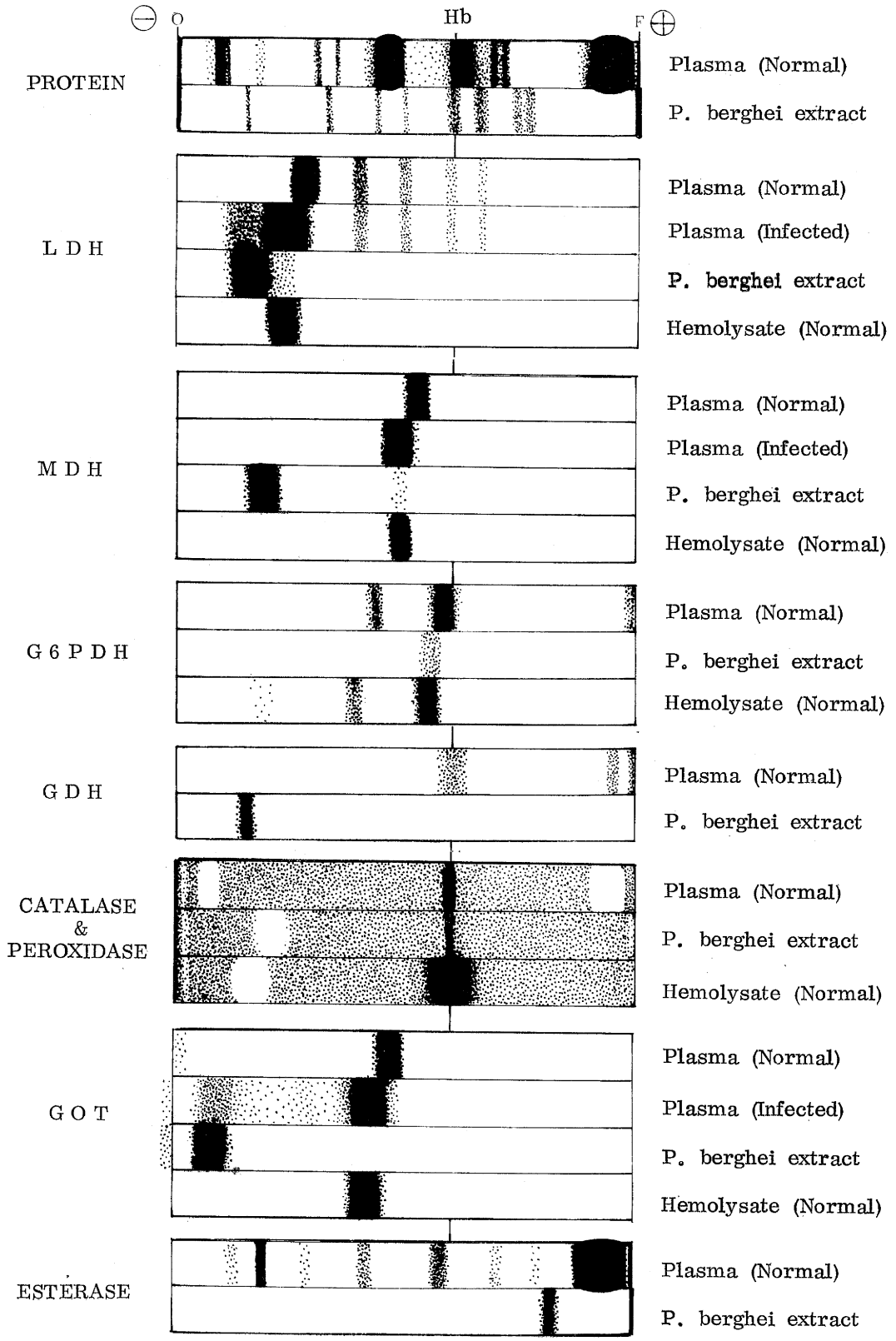

Fig. 2. Comparison of electrophoretic patterns for protein and enzyme bands in *Plasmodium berghei*, normal and malaria-infected mouse plasma (O : origin, F : buffer front).

experimental conditions fortified with NADP and $MnCl_2$, no "malic enzyme" band was detected both in mouse plasma and in the parasite extracts.

G6PDH

Usually 3 G6PDH isoenzyme bands could be detected in plasma samples obtained from uninfected mice : one at the transferrin position, other just at the Hb band, and anothor near the buffer front. Malaria-infected mice usually showed plasma G6PDH pattern where the 2nd band was more intense in color after staining for the enzyme activity; this might be due to hemolysis by the heavy infection because this intense band corresponded to that found in RBC hemolysates. Cell-free extracts of the parasites possessed lower G6PDH activity than that of plasma, and only one activity band had been detected of which position corresponded to the host RBC G6PDH band at just below the Hb band.

GDH

At least 3 GDH isoenzymes could be detected in mouse plasma, whereas no activity band could be found in hemolysates of normal mouse RBC. The soluble extracts of malaria parasites possessed at least one GDH band, which migrated very slowly (slightly lower than plasma ChE) and no corresponding band was shown in the host blood materials, indicating that this GDH band was specific to the malaria parasite. Several GDH's which require different coenzymes are known in animals and plants. In this malaria species, NADP was an effective coenzyme whereas NAD was not; only very weak band could be detected with NAD. Thus the parasite-specific GDH seems to be an NADP-dependent GDH: EC 1.4.1.4.

Catalase & Peroxidase

There were no remarkable qualitative differences in isoenzyme patterns of plasma or RBC between normal mice and malaria-infected ones, though malaria-parasitized RBC showed rather strong catalase activity. Cell-free extracts of the malaria parasite had a single catalase band of which mobility was slightly faster than that of RBC hemolysates, and no corresponding catalase band was detected in the host plasma samples. No specific peroxidase band other than the Hb was found in infected mouse blood or malaria parasite extracts.

GOT

Normal mouse plasma showed one major enzyme activity band at the transferrin area and a lower activity band close to the origin. Normal RBC possessed a single major GOT band which migrated rather slowly than the major band found in plasma. Plasma samples obtained from malaria-infected mice showed a rather single intense band which covered GOT band areas of both plasma and RBC. Sometimes the slowest band near the origin showed heavy color intensity. Extracts of the host cell-free parasites demonstrated an obviously colored band close to a transparent band which located just adjacent to the

anodal side of the origin. An additional band which slightly migrated towards the cathode was also observed. Thus plasma samples taken from mice with heavy parasitemia showed a higher GOT activity of both parasitic and host origins.

Esterases

There are many variations of esterase isoenzymes in activity or in number of bands among mice, gel concentration, and substrate used (Hunter and Strachan, 1961). When α-naphthyl acetate is used as a substrate, 7–9 bands of so-called non-specific esterases can be distinguished in normal mouse plasma. The major esterase bands are an albumin-associated arylesterase and a plasma ChE. Between these 2 major bands, usually 3–5 esterase bands can be observed.

Cell-free extracts of parasites showed a single major esterase band, of which position corresponded to a post-albumin area where no major esterase bands could be observed in normal mouse plasma, WBC or RBC. Thus this esterase seems to be a parasite-specific enzyme.

GENERAL CONSIDERATION

It is well known that the host RBC is rich in enzymes of both glycolysis and pentose phosphate cycle. The host cell-free *P. berghei* also can metabolize ^{14}C-labeled glucose predominantly to lactate (Bowman *et al.*, 1961; Bryant *et al.*, 1964), indicating the possession of a complete set of the enzymes in glycolytic pathway in this malarial species. Electrophoretically, up to the date, at least 2 enzymes of glycolysis have been demonstrated to occur in *P. berghei*: glucose phosphate isomerase, EC 5.3.1.9 (Carter, 1970; Walliker *et al.*, 1971) and LDH (Sherman, 1962; Nagarajan, 1968; Phisphumvidhi and Langer, 1969). Results of the present work also confirm the presence of the parasite-specific LDH.

Bryant *et al.* (1964) have shown that radioactivity of ^{14}C-glucose is also incorporated, in lesser extent, into aspartate, glutamate and alanine in free parasites, suggesting the presence of a transamination system which is connected to the glycolysis. The result reported here evidently demonstrates the possession of GOT of parasitic origin. Electrophoretic demonstration of GPT in the extracts of *P. berghei*, however, has not been successful yet.

For these years there have been some confusions on whether or not malaria parasites possess the Krebs' TCA cycle, but it is now recognized that erythrocytic forms of mammalian malaria parasites cannot metabolize pyruvate into citrate via acetyl-CoA and lack several members of the TCA cycle whereas avian malarial species seem to possess the TCA cycle (Bryant and Voller, 1961; Nagarajan, 1968; Peters, 1969, 1970). The presence of only 2 enzymes of the TCA cycle has been reported in *P. berghei*: SDH (Nagarajan, 1968; Howells *et al.*, 1970) and MDH (Sherman, 1966, Nagarajan, 1968). How to connect the glycolysis to the TCA cycle or to the transamination in this malaria parasite metabolism is still wrapped in mystery, although Nagarajan (1968, III) have

discussed on several possible pathways. At least under the experimental conditions employed, no detectable NADP-malic enzyme pathway has been found in both parasite extracts and the host blood components in accordance with results obtained by Nagarajan (1968, I). Thus speculations by other plausible pathways, especially CO_2 fixation from phosphoenolpyruvate to oxaloacetate or from pyruvate to oxaloacetate, await to be demonstrated directly.

Another example of confusions on the glucose metabolism is whether or not *P. berghei* possesses its own G6PDH which acts as a key enzyme to the pentose phosphate cycle. Sherman (1965) has reported that when parasitemia beyond 78% the G6PDH deficiency was observed in *P. berghei*-infected mouse RBC. Working on free parasite extracts of this species, Langer *et al.* (1967) have demonstrated the existance of the parasite-specific G6PDH and 6PGDH which differ in electrophoretic mobility from those of the host RBC. On the contrary, Theakston and Fletcher (1971) have reported, using the N strain of *P. berghei* and electron cytochemical techniques, that no G6PDH activity was observed within the parasite cytoplasm except for food vacuoles. More recently, according to Theakston and Fletcher (1973), their unpublished data obtained by electrophoresis on Cellogel have shown the presence of only the erythrocytic G6PDH band in the extracts of the malaria parasite used. Result of the present work with the NK 65 strain of *P. berghei* is also in accordance with this observation. One of the plausible explanations to understand such a discrepancy may be the strain-specific differences (*i. e.*, genetic variations) within a species or even within a subspecies. Carter (1970) has beautifully demonstrated an example of such electrophoretic variations of several enzymes in various strains of *P. berghei* (s. l.), and Howells *et al.* (1970) have shown a significant difference in the presence of SDH activity between chloroquine sensitive N strain and resistant RC and NS lines. Furthermore, enzyme activity may or may not be detectable during the parasite's life cycle (Howells, 1970). Unfortunately, Langer *et al.* (1967) did not mention the name of the parasite strain used, and Carter (1970) did not report any about G6PDH isoenzymes. Therefore, it is still uncertain whether the G6PDH band found in the parasite extracts is truly originated from the malaria parasite or is just derived from an ingested host RBC material within the parasite "food vacuoles".

Presence of the NADP-dependent GDH in this malaria parasite has been reported enzymologically by Langer *et al.* (1970) while they did not mention about its electrophoretic characteristics. Results of the present work thus electrophoretically confirm the evidence that the parasitic GDH is distinct from the host enzyme. It is generally recognized by some workers that GOT and GPT levels in blood serum or plasma significantly increase in malaria-infected rodents (Sadun *et al.*, 1965, 1966; Wellde *et al.*, 1966). However, there was no experimental proof to distinguish whether such increases in GOT level being derived from disintegrated RBC or from the parasite. The present work reported here again evidently demonstrates that both the host enzyme and the parasitic one can be attributed to the increased enzyme level in plasma. It is now interesting to know that the reaction between glutamate and 2-oxoglutarate is catalyzed

by both the transaminase and the dehydrogenase, suggesting an important role of these compounds in the metabolism of *P. berghei*.

In conclusion, electrophoretic comparison seems to be one of the most effective resorts, at present, to diagnose parasite-specific enzymes from those of host origin.

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REFERENCES

1) Allen, J. M. (1961): Multiple forms of lactic dehydrogenase in tissues of the mouse: Their specificity, cellular localization, and response to altered physiological conditions. Ann. N. Y. Acad. Sci., 94, 937–951.

2) Bowman, I. B. R., Grant, P. T., Kermack, W. O. & Ogston, D. (1961): The metabolism of *Plasmodium berghei*, the malaria parasite of rodents. 2.An effect of mepacrine on the metabolism of glucose by the parasite separated from its host cell. Biochem. J., 78, 472–478.

3) Bryant, C. & Voller, A. (1961): Preliminary studies on carbon utilization patterns of *Plasmodium vinckei*. Trans. Roy. Soc. Trop. Med. Hyg., 55, 305.

4) Bryant, C., Voller, A. & Smith, M.J.H. (1964): The incorporation of radioactivity from [^{14}C] glucose into the soluble metabolic intermediates of malaria parasites. Amer. J. Trop. Med. Hyg., 13, 515–519.

5) Carter, R. (1970): Enzyme variation in *Plasmodium berghei*. Trans. Roy. Soc. Trop. Med. Hyg., 64, 401–406.

6) Chavin, S. I. (1966): Studies on the antigenic constituents of *Plasmodium berghei*. Milit. Med., 131, 1124–1136.

7) Corradetti, A., Verolini, F., Bucci, A. & Pennacchio, A. E. (1971): Elettroforesi a disco di due estratti idrosolubili di plasmodi : *Plasmodium berghei* (ceppo Istisan) e *Plasmodium berghei* (ceppo Vincke 1967). Parasitologia, 13, 385–389.

8) Davis, B. J. (1964): Disc electrophoresis– Ⅱ .Method and application to human serum proteins. Ann. N. Y. Acad. Sci., 121, 404–427.

9) Dersowitz, R. S., Saave, J. J. & Stein, B. (1966): The application of the indirect haemag-

glutination test in recent studies on the immuno-epidemiology of human malaria and the immune response in experimental malaria. Milit. Med., 131, 1157–1166.

10) Dietz, A. A., Lubrano, T. & Rubinstein, H. M. (1970): Disc electrophoresis of lactate dehydrogenase isoenzymes. Clin. Chim. Acta, 27, 225–232.

11) Fulton, J. D. & Grant, P. T. (1956): The sulphur requirements of the erythrocytic form of *Plasmodium knowlesi*. Biochem J., 63, 274–282.

12) Goldberg, E. (1965): Lactate dehydrogenase in spermatozoa: subunit interaction *in vitro*. Arch. Biochem. Biophys., 109, 134–141.

13) Howells, R. E. (1970): Mitochondrial changes during the life cycle of *Plasmodium berghei*. Ann. Trop. Med. Parasit., 64, 181–187.

14) Howells, R. E., Peters, W., Homewood, C. A. & Warhurst, D. C. (1970): Theory for the mechanism of chloroquine resistance in rodent malaria. Nature, 228, 625–628.

15) Hunter, R. L. & Strachan, D. S. (1961): The esterases of mouse blood. Ann. N. Y. Acad. Sci., 94, 861–867.

16) Langer, B. W., Jr., Phisphumvidhi, P. & Friedlander, Y. (1967): Malarial parasite metabolism: The pentose cycle in *Plasmodium berghei*. Exp. Parasit., 20, 68–76.

17) Langer, B.W.,Jr.,Phisphumvidhi, P. & Jiampermpoon,D. (1970): Malarial parasite metabolism: The glutamic acid dehydrogenase of *Plasmodium berghei*. Exp. Parasit., 28, 298–303.

18) Nagarajan, K. (1968): Metabolism of *Plasmodium berghei*. I. Krebs cycle; III. Carbon dioxide fixation and role of pyruvate and dicarboxylic acids. Exp. Parasit., 22, 19–26; 33–42.

19) Ogita, Z. (1965): Polyacrylamide gel elcctrophoresis. I. Metab. Dis., 2, 331–334. (in Japanese)

20) Peters, W. (1969): Recent advances in the physiology and biochemistry of plasmodia. Trop. Dis. Bull., 66, 1–29.

21) Peters, W. (1970): Chemotherapy and drug resistnce in malaria, 34–43. Acad. Press, London & N. Y.

22) Phisphumvidhi, P. & Langer, B. W., Jr. (1969): Malarial parasite metabolism : The lactic acid dehydrogenase of *Plasmodium berghei*. Exp. Parasit., 24, 37–41.

23) Richards, W. H. G. & Williams, S. G. (1973): The removal of leucocytes from malaria infected blood. Ann. Trop. Med. Parasit., 67, 249–250.

24) Sadun, E.H., Williams, J. S. & Martin, L.K. (1966): Serum biochemical changes in malarial infections in men, chimpanzees and mice. Milit. Med., 131, 1094–1106.

25) Sadun, E. H., Williams, J. S., Meroney, F. C. & Hutt, G. (1965): Pathophysiology and *Plasmodium berghei* infection in mice. Exp. Parasit., 17, 277–286.

26) Sherman, I. W. (1962): Heterogeneity of lactic dehydrogenase in intra-erythrocytic parasites. Trans. N. Y. Acad. Sci. II, 24, 944–953.

27) Sherman, I. W. (1965): Glucose-6-phosphate dehydrogenase and reduced glutathione in malaria-infected erythrocytes (*Plasmodium lophurae* and *P. berghei*). J. Protozool., 12, 394–396.

28) Sherman, I. W. (1966): Malic dehydrogenase heterogeneity in malaria (*Plasmodium lophurae* and *P. berghei*). J. Protozool., 13, 344–349.

29) Sizaret, P., O'Conor, G. T., Beaumont, R. & Laval, M. (1971): Serum protein patterns in mice following primary and challenge infection with *Plasmodium berghei*. Zeit. Tropenmed. Parasit., 22, 260–270.

30) Spira, D. & Zuckerman, A. (1966): Recent advances in the antigenic analysis of plasmodia. Milit. Med., 131, 1117–1123.

31) Tella, A. & Maegraith, B. G. (1965): Physiopathological changes in primary acute blood-transmitted malaria and *Babesia* infections. II. — A comparative study of serum-protein levels in infected rhesus monkeys, mice and puppies. Ann. Trop. Med. Parasit., 59, 153−158.

32) Theakston, R. D. G. & Fletcher, K. A. (1971): An electron cytochemical study of glucose-6-phosphate dehydrogenase activity in erythrocytes of malaria-infected mice, monkeys and chickens. Life Sci., 10, 701−711.

33) Theakston, R. D. G. & Fletcher, K. A. (1973): An electron cytochemical study of 6-phosphogluconate dehydrogenase activity in infected erythrocytes during malaria. Life Sci., 13, 405−410.

34) Walliker, D., Carter, R. & Morgan, S. (1971): Genetic recombination in malaria parasites. Nature, 232, 561−562.

35) Wellde, B. T., Briggs, N. T. & Sadun, E. H. (1966): Susceptibility to *Plasmodium berghei*: Parasitological, biochemical and hematological studies in laboratory and wild mammals. Milit. Med.,131, 859−869.

ポリアクリルアミドゲル電気泳動によるネズミマラリア原虫特異酵素の検出

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マラリア原虫を感染させたマウスでは正常マウスに較べ血漿の総蛋白質量，アルブミン量などが低下する傾向が見られたが，一方乳酸脱水素酵素やアミノ転移酵素などの活性は著しく上昇した．その原因が感染に伴なう宿主血液系における変化によるものであるのか，あるいは寄主自身の産生する酵素によるものであるのかはこれらの測定値だけからは区別することができない．そこでマラリアの生化学とくに寄主宿主関係についての基礎的知見を得るためにポリアクリルアミドゲルによる電気泳動を行ない，正常マウス，マラリア感染マウスの血漿，赤血球，分離マラリア原虫などの可溶性蛋白質や酵素の泳動像について比較検討を試みた．その結果，乳酸脱水素酵素，リンゴ酸脱水素酵素，グルタミン酸脱水素酵素，カタラーゼ，グルタミン酸オキザロ酢酸アミノ転移酵素，エステラーゼなどにおいてマラリア原虫に特有と考えられる活性泳動帯が認められた．また感染マウス血漿で著しい活性上昇が見られた乳酸脱水素酵素やグルタミン酸オキザロ酢酸アミノ転移酵素では，赤血球の崩壊に伴なう宿主側の酵素とマラリア原虫自身に由来する酵素の双方がその原因となっていることが証明された．マラリア原虫の解糖系に隣接する代謝系についての報告，とくにブドウ糖６燐酸脱水素酵素の有無については，従来多くの混乱が見られたが，今回用いられた系統のマラリア原虫抽出液中には宿主赤血球の酵素と一致する活性帯しか検出されなかった．このように同一ゲル上での電気泳動による比較は寄主宿主間の酵素系の異同を識別する上で極めて有力な手段であることが示された．

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